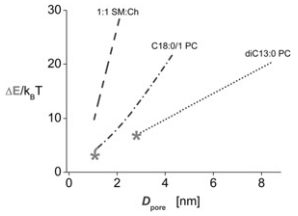
tension, covering rates from < 0.01/s to > 100/s. Plotted as logarithms, rates of nanopore nucleation yield changes in activation energy (scaled by thermal energy k_BT). Derivatives of activation energy with respect to tension establish the critical-pore areas that couple with tension to lower activation energy. When correlated to pore area, changes in activation energy can be transformed to free energies along a reaction coordinate defined by pore size. In this way, we have mapped free-energy landscapes for pore nucleation in several types of fluid-lipid bilayer systems (cf. figure), beginning near the symmetry break and rising by 10-20 k_BT when pore sizes reach a few nm. [E. Evans and B.A. Smith, *New Journal of Physics* **13** (2011)].



1179-Symp
What is a Membrane? Mechanical Stress Viewed with the Patch Clamp
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Much of what we know about cell membranes has come from studies of purified lipids in various forms: monolayers, bilayers, etc. However, cell membranes are much more complicated and even the definition of a cell membrane fails when we address the mechanics. Where does the membrane start and where does it end? In most cells, stresses are distributed in three dimensions and include the extracellular matrix, the bilayer and the cytoskeleton. What are we discussing when we refer to membrane stress? In bacteria, mechanosensitive ion channels (MSCs) have been reconstituted into lipid membranes so that the relevant stress is tension in the bilayer: a mean value or stress in a sub domain such as the head groups. Lipid reconstitution has yet to be verified for eukaryotic MSCs. If we assume that lipid stress adjacent to the channel is the driving stimulus, then for cells we need a relationship between the applied stimulus and the local stimulus. The applied stimulus is commonly a hydrostatic pressure across the bilayer produced by suction on a patch pipette. If the patch were elastic it would respond at the same rate as the pressure, but it is much slower (~ 100ms) when judged by MSC activity or patch capacitance. This viscosity is probably generated by bond breaking in the stressed cytoskeleton that bears a substantial amount of the mean tension. Thus, patch mechanics are governed by multiple forces including hydrostatics, viscoelastic properties of the cytoskeleton and lipid, and the energy of adhesion of the membrane to the glass. Caution is advised in the interpretation mechanics data from a patch.

1180-Symp
Proteins Shaping Membranes : Quantitative Measurements
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Membrane transport between intracellular compartments, entry or exit out of the cell, imply similar sequential events: membrane deformation and lipid/protein sorting during the formation of the transport intermediate (vesicle or tube), fission from the donor compartment, transport and eventually fusion with the acceptor membrane. The mechanisms behind these biological processes of membrane transformation are actively studied both in the cell biology and the biophysics contexts. Membrane nanotubes with a controlled diameter (15-500 nm) pulled out of Giant Unilamellar Vesicles (GUV) are very convenient tools to address the role of curvature in trafficking events and to measure mechanical effects due to protein binding using optical tweezers.
As an example of this type of approach combining in vitro experiments and theoretical modeling, I will present our results on two proteins implied in clathrin-mediated endocytosis. Amphiphysin 1 contains a N-BAR membrane-binding domain. We have shown that at low protein density on the GUV, the distribution of proteins and the mechanical effects induced are well described by a model based on spontaneous curvature induction. At high densities, the radius and force are independent of tension and vesicle protein density, resulting from the formation of a scaffold around the tube. For the entire density range, protein was found to be enriched on the tube as compared to the GUV, showing a concomitant curvature-sensing ability. I will compare this behavior with that of another protein, the dynamin, induced the scission of the clathrin-coated vesicle in cells. In contrast, there is a threshold for the tube radius above which no binding occurs, but below which dynamin forms a scaffold constricting the tube.

1181-Symp
New Insights into the Formation and Function of Caveolae
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One of the most abundant and characteristic surface microdomains of mammalian cells seen by electron microscopy are surface pits termed caveolae. Despite their abundance the exact functions of caveolae remain elusive. Over the last ten years our research has focussed on the fine ultrastructure of caveolae, the major components of caveolae, and their specific cellular functions. Caveolins, abundant membrane proteins of caveolae, play a crucial role in the formation of caveolae. Mutations in caveolins are associated with breast cancer and with a number of muscle diseases, including limb girdle muscular dystrophy. Our recent studies, utilising a range of systems including mouse models, cultured cells, and the zebrafish embryo, have identified a new family of coat proteins which regulate caveola formation. PTRF-cavin family members regulate association of caveolin with caveolae and identify a cellular mechanism to regulate caveolar and non-caveolar functions of caveolins. These studies have implications for understanding the distinct cell-type specific roles of caveolin in disease conditions. We are also studying how caveolin-lipid interactions generate the unique architecture of the caveolar domain by studying caveola formation in caveolin-null fibroblasts and in a model system. By combining these studies with high resolution electron microscopy of fast frozen freeze-substituted caveolae in vitro and in vivo and with tomography of cryofixed vitrified material, we have gained fundamental new insights into the molecular interactions involved in caveola formation. These results not only have implications for the understanding of caveola dysfunction in disease but also provide general insights into the mechanism by which a single membrane protein can generate the membrane curvature characteristic of caveolae. In addition, our studies on caveolins and cavins are providing insights into a novel role for caveolae as mechanosensory organelles.

Platform: Member-organized Session: Förster Resonance Energy Transfer: Spectroscopy & Microscopy

1182-Plat
Single-Molecule FRET in Living Bacteria
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Most single-molecule FRET experiments are performed in vitro, using tightly controlled conditions and well-defined concentrations of a limited number of interacting components. However, in order to understand biological mechanisms as they occur in vivo while simultaneously taking advantage of the extra information provided by single-molecule detection, there is a growing need for performing single-molecule fluorescence measurements in cellular contexts, and in particular in living cells.
Towards this goal, we have developed physical methods for delivering fluorescent biomolecules in living Escherichia coli bacteria (one of the most common model organisms in biology) and observing single-molecule fluorescence and single-molecule FRET in the bacterial cytoplasm; we use both confocal and wide-field imaging approaches for detection, providing access to a large number of probed timescales. We have also been using localization-based super-resolution imaging approaches to study the subcellular localization, mobility and abundance of the internalized biomolecules.
Our results using single-stranded and double-stranded DNA standards with different FRET efficiencies show that the FRET efficiency of the internalized DNAs agrees well with in vitro FRET measurements. Single-molecule FRET time-traces from the majority of internalized molecule show the characteristic spectroscopic signatures expected from a single FRET pair system. Ongoing work on other biomolecules, including doubly labelled proteins, should lead to the exciting prospect of visualizing sub-nanometer conformational changes at the single-molecule level in the natural milieu of live cells. Our approaches are general and should be useful for studying a large number of intracellular processes in bacteria.

1183-Plat
Analysis of FRET Biosensor Distribution in 3D by the Phasor Approach to FLIM
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The phasor approach to analyze FLIM images has several advantages with respect to the classical multi exponential of the decay at each pixel of an image. More importantly, the phasor approach lends itself to simultaneous correlation

of lifetime changes with spatial localization. Here we describe an application of FRET image analysis using the Rac and RhoA biosensors in which the specific distribution of the sensor in a cell is important to establish its activation. Specifically we study cells in a 3D matrix in which the activation of the Rac and RhoA biosensor could have a different distribution than in 2D. In order to simultaneously measure several 3D locations we use a method in which we measure FRET along a 3-dimensional line which encompasses different parts of the cell. The measurement of the decay at each point of a 3D line can be done very fast (in millisecond) potentially revealing the dynamics of the biosensor at a time scale that is of particular significance for cellular reactions. When the laser spot is moving along the line, we can also do measurements at two emission wavelengths giving us the chance to compare the phasor-FRET determination with the ratiometric method.
The authors acknowledge financial support from the National Institutes of Health P50 GM076516 and P41 RR03155

1184-Plat
Polarized Fluorescence Correlation Spectroscopy (pFCS): A Single-Molecule Method for Simultaneously Measuring Homo-FRET, Brightness, and the Diffusion of Protein Complexes in Living Cells
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FRET is a phenomenon where excited state energy of a fluorescent donor molecule is transferred, by a non-radiative dipole-dipole coupling mechanism, to a nearby acceptor. Because the efficiency of FRET provides information regarding the distance separating the donor and acceptor, FRET is used to study protein interactions in living cells. In conjunction with automated microscopy, FRET can also be used to screen for drugs that perturb specific protein-protein interactions. The utility of FRET, however, is limited by the possibility of both false positive (caused by over expression and molecular crowding), and by false negative signals (resulting from separation distances greater than 10 nm and from very low dipole orientation factors). To overcome these limitations we have developed Polarized Fluorescence Correlation Spectroscopy (pFCS), a single-molecule based method to characterize the interactions of proteins in complexes. Using pFCS, Homo-FRET (a 1-10 nm proximity gauge), brightness (a measure of the number of fluorescent subunits in a complex), and the lateral diffusion coefficient (an attribute sensitive to viscosity, mass, and the shape of a protein complex) can be simultaneously measured. With these measurements, the interpretation of FRET can be rigorously constrained thus reducing the likelihood of both false negative and false positive interpretations. Standards consisting of tandem covalently linked concatemers of between 1 and 6 Venus molecules were used to validate pFCS in both solution and in cells. The utility of pFCS was demonstrated by measuring differences in Homo-FRET, subunit stoichiometry and correlation time between Venus-tagged CaM-kinase-II holoenzyme (α or β).

1185-Plat
Dynamics of DNA Mismatch Repair Revealed by Single Molecule FRET
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Single molecule FRET (smFRET) has rapidly gained popularity because it can provide unique information about biomolecular systems. In particular, smFRET provides quantitative nanoscale resolution of dynamic molecular motions and multimolecular interactions, all for unsynchronizable samples that may have multiple reaction pathways operating in parallel. As the experimental capabilities of smFRET advance, this method has been applied to increasingly complex biological systems. I will illustrate these complex system advantages of single molecule FRET by presenting measurements of DNA mismatch repair proteins MutS and MutL interacting with mismatched DNA. These proteins are the initial sentries that detect single base mismatches and insertions/deletions and activate repair cascades. We use smFRET to determine dynamic DNA bending by MutS, concomitant conformational changes within MutS itself, motion of MutS scanning along DNA, ATP binding states that commit MutS:mismatch DNA complexes to convert to sliding states used in signaling, and the modulation of these MutS behaviors by interactions with MutL.

1186-Plat
Single-Molecule FRET: Theory and Analysis of Photon Sequences
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Single-molecule FRET measurements contain information on conformational dynamics because the rate of energy transfer depends on the distance between donor and acceptor labels attached to a molecule. The output of such measurements is a sequence of photons of different colors separated by apparently random time intervals. In addition, the delay time between laser pulse and photon arrival can be recorded. To extract information from such raw data, it is necessary to understand in detail all the complex microscopic processes involved. We consider various quantitative methods to analyze sequences of photons emitted by a molecule with interchanging conformational states. Photon sequences with recorded interphoton times can be analyzed by maximizing the appropriate likelihood functions with respect to the parameters of a model of the conformational dynamics. The consistency of the model with the data can be checked by recoloring the photons trajectory and comparing the predicted and observed FRET efficiency histograms. These photon-by-photon methods are rigorous for both immobilized and diffusing molecules. Binned photon sequences, in which only the numbers of donor and acceptor photons in consecutive time intervals are recorded, can be analyzed by constructing FRET efficiency histograms or, alternatively, by analyzing the whole sequence of photon counts using likelihood-based methods. For the FRET efficiency histograms, we derive accurate multi-Gaussian approximations without any adjustable parameters when the molecule has multiple conformational states. For the whole sequence analysis, we provide approximate likelihood functions for the binned photon sequences. It is shown how these methods can be extended to include information from photon delay times.

1187-Plat
Structural Modeling of Full Length Maguk Scaffold Proteins using Single Molecule FRET Restraints
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By conjoining protein interaction domains, scaffold proteins form a framework to organize signaling. Synaptic MAGUK scaffold proteins contain three PDZ domains, an SH3 domain and an inactive Guanylate kinase (GK) domain, which are connected by flexible linkers. We used single molecule fluorescence to probe the structure of MAGUK proteins in their isolated ''ground'' state. We found that the five domains partition into two independent units with relatively fixed structures. From these findings, it is unclear how allosteric coupling between the PDZ domains would be possible. Comparative structural analysis showed a conservation of the PDZ domain organization but the differences within the PDZ3-SH3-GK module. Surprisingly, we found no signs of conformational heterogeneity, transitions or dynamics on the microsecond to second timescale. These findings provide the first unambiguous assignment of domain positioning in a full length scaffold protein.

1188-Plat
From Force-Fields to Photons: MD Simulations of Dye-Labeled Nucleic Acids and Monte Carlo Modeling of FRET
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Fluorescence resonance energy transfer (FRET) is a powerful technique for understanding the structural fluctuations and transformations of RNA, DNA and proteins. Molecular dynamics (MD) simulations provide a window into the nature of these fluctuations on a different, faster, time scale. We use Monte Carlo methods to model and compare FRET data from dye-labeled RNA with what might be predicted from the MD simulation. With a few notable exceptions, the contribution of fluorophore and linker dynamics to these FRET measurements has not been investigated. We include the dynamics of the ground state dyes and linkers in our study of a 16mer double-stranded RNA. Water is included explicitly in the simulation. Cyanine dyes are attached at either the 3' or 5' ends with a 3 carbon linker, and differences in labeling schemes are discussed.

1189-Plat
High Precision FRET to Determine Dynamic Protein Structures
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Measuring in solution and utilizing the single-molecule advantage of fluorescence detection we established a toolbox to generate FRET-constrained structure models of biomolecules which can also show their heterogeneity and flexibility. Our approach comprises seven steps: **(1)** Quantitative measurement of FRET by multiparameter fluorescence detection of single molecules [1]; (2) Rigorous analysis and error determination of FRET derived donor-acceptor distances by analyzing the photon distributions and time resolved anisotropies of